Expression of Insulin-Like Growth Factor I by Cultured Skin Substitutes Does Not Replace the Physiologic Requirement for Insulin *In Vitro*

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Clinical efficacy of cultured skin substitutes may be increased if their carbohydrate metabolism is optimized by understanding whether endogenous insulin-like growth factor I can substitute for exogenous insulin. Cultured skin substitutes were prepared and incubated at the air–liquid interface for 4 wk in media containing 0.5 or 5 μg per ml insulin, 10 or 50 ng per ml insulin-like growth factor I, or 0 insulin and 0 insulin-like growth factor I (negative control). *In situ* hybridization showed that the epidermal and dermal cultured skin substitute components express insulin-like growth factor I mRNA throughout the 28 d interval. Immunohistochemistry confirmed the expression of insulin-like growth factor I protein by the human keratinocytes and fibroblasts in cultured skin substitutes. Insulin-like growth factor I at 10 or 30 ng per ml could partially replace insulin in a clonal assay of keratinocyte growth. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays showed significantly higher values in cultured skin substitutes incubated with insulin at incubation days 14 and 28 compared to negative control or the 10 ng per ml insulin-like growth factor I condition. Cultured skin substitutes incubated in 50 ng per ml insulin-like growth factor I had MTT values similar to the insulin-treated cultured skin substitutes at day 14, but were significantly lower by day 28. Light microscopy agreed with MTT data showing that cultured skin substitutes grown with insulin media had multiple layers of nucleated keratinocytes and stratum corneum at days 14 and 28. The negative control and 10 ng per ml insulin-like growth factor I exhibited poor cultured skin substitute epidermal morphology throughout the experiment. In contrast, the cultured skin substitutes in 50 ng per ml insulin-like growth factor I were similar to the insulin-treated cultured skin substitutes at day 14, but by day 28 had deteriorated to resemble the negative control. Bromodeoxyuridine incorporation at day 28 was significantly higher for 5 μg per ml insulin cultured skin substitutes *versus* all other treatment groups. These data suggest that medium containing 5 μg per ml insulin supports greater physiologic stability in cultured skin substitutes over time, and that expression of insulin-like growth factor I by keratinocytes and fibroblasts in cultured skin substitutes is not sufficient to fully replace the requirement for exogenous insulin *in vitro*.

Key words: DNA synthesis/epidermal barrier/fibroblasts/keratinocytes/wound healing. J Invest Dermatol 116: 650–657, 2001

Cultured skin substitutes (CSS) have become adjunctive therapies for treatment and healing of full-thickness skin wounds (Boyce, 1996). Epidermal barrier is the definitive property of wound closure, and consists of a variety of lipids that are synthesized in keratinocytes from two-carbon subunits produced by catabolism of carbohydrates (Elias, 1983; Ponec *et al*, 1997). Therefore, formation of epidermal barrier depends on carbohydrate metabolism of keratinocytes. Carbohydrate uptake is regulated primarily by extracellular insulin, which is delivered from the plasma *in vivo* and is a universal requirement for serum-free culture of nontransformed human cells (Ham and McKeehan, 1979). It is also known that insulin-like growth factor I (IGF-I) supports proliferation of nontransformed cells in culture, that it can bind to the insulin receptor (LeRoith *et al*, 1994), and that it is mitogenic for both keratinocytes and fibroblasts in culture (Conover *et al*, 1985; Nickoloff *et al*, 1988; Ristow and Messmer, 1988; Barreca *et al*, 1992; Kratz *et al*, 1992). It is not clear whether IGF-I can substitute for insulin in the proliferation and differentiation of keratinocytes and fibroblasts incubated to generate CSS, however, nor whether autocrine and paracrine release of IGF-I from cells in CSS can reduce or eliminate the requirement for exogenous insulin.

Manuscript received October 19, 2000; revised January 25, 2001; accepted for publication January 29, 2001.

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Abbreviations: CSS, cultured skin substitute; DIG, digoxigenin; GAG, glycosaminoglycan; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; SEC, surface electrical capacitance.

IGF-I is a 70 amino acid peptide that has approximately 50% structural homology to proinsulin (Daughaday and Rotwein, 1989). Generally, intermediary metabolism is regulated by insulin via the insulin receptors on liver, fat, and muscle cells. In contrast, IGF-I is ascribed the function of growth by acting through the type I IGF receptors found on most cells (LeRoith *et al*, 1994). IGF-I is found in human serum associated with one of its binding proteins and it is responsible for the somatotropic effects of growth hormone (Clark, 1997). Insulin and IGF-I have structurally homologous high affinity receptors in most tissues including the human epidermis and dermis (Nickoloff *et al*, 1988; Krane *et al*, 1991; Neely *et al*, 1991; Tavakkol *et al*, 1992; LeRoith *et al*, 1994). IGF-I and insulin can bind to each other's respective receptor, but with lower affinity (Neely *et al*, 1991). High doses of insulin bind the type I IGF receptor and can mimic the activity of IGF-I in epidermal cell culture medium (Ristow and Messmer, 1988; Aaronson *et al*, 1990; Neely *et al*, 1991). Cultured human fibroblasts and melanocytes, but not human keratinocytes, have been reported to produce IGF-I (Tavakkol *et al*, 1992, 1999), although upon ultraviolet B irradiation IGF-I immunoreactivity was observed in the mouse epidermis (Hansson *et al*, 1988) and the healing epithelial edge of full-thickness cutaneous wounds expressed IGF-1 protein and mRNA in mice (Brown *et al*, 1997). In addition, the differentiated human keratinocytes of the stratum granulosum and dermal fibroblasts were positive for IGF-I by *in situ* hybridization (Rudman *et al*, 1997), and by immunohistochemistry in all layers of uninjured human epidermis (Blakytny *et al*, 2000).

The function of IGF-I has been investigated in wound healing. IGF-I mRNA levels were elevated in rat wounds with no change in IGF-I receptor expression (Gartner *et al*, 1992; Steenfos and Jansson, 1992). Wound repair was significantly accelerated when mouse wounds were treated with a combination of IGF-I and IGF binding protein I (Tsuboi *et al*, 1995). Wounds created on human skin cultured *in vitro* re-epithelialized at a greater rate in the presence of IGF-I (Kratz *et al*, 1994). Lynch *et al* (1989) found that a combination of platelet-derived growth factor 2 and IGF-I stimulated the epidermal and dermal development in porcine partial-thickness wounds. Recombinant human growth hormone when delivered by subcutaneous injection resulted in improved healing times of graft donor sites for burn wounds presumably by induction of IGF-I (Herndon *et al*, 1995). Burn patients with large burns had lower IGF-I serum levels that correlated with the level of serum albumin (Moller *et al*, 1991). This reduced IGF-I concentration may be responsible in part for the reduced healing observed in these patients. In the postburn hypertrophic scar, elevated levels of IGF-I and type I collagen were measured (Ghahary *et al*, 1995). In a subsequent study, fibroblasts derived from the hypertrophic scars synthesized less collagenase mRNA when treated with IGF-I, but increased type I collagen production *in vitro* (Ghahary *et al*, 1996). Thus, IGF-I may positively influence wound healing, but IGF-I overexpression may have deleterious consequences.

Previous studies from this laboratory have demonstrated closure of large burns and chronic wounds with CSS (Boyce *et al*, 1995a, b, 1999; Harriger *et al*, 1995). CSS are composed of autologous human keratinocytes and fibroblasts inoculated onto a collagen-glycosaminoglycan (GAG) biopolymer substrate (Boyce and Hansbrough, 1988; Boyce, 1998). CSS have been shown to generate epidermal barrier *in vitro* that contributes to more rapid wound closure (Boyce *et al*, 1996; Supp *et al*, 1999). Medium containing insulin supports the formation of epidermal barrier with simultaneous maintenance of proliferation of keratinocytes and fibroblasts (Boyce *et al*, 1997). Preliminary studies suggested that endogenous IGF-I in the absence of insulin may support keratinocyte metabolism and limited differentiation (Swope *et al*, 1997a, b). The studies reported here were designed to investigate whether endogenous or exogenous IGF-I can support the proliferation and differentiation of keratinocytes and fibroblasts in CSS, and whether IGF-I is expressed in the epidermal and/or dermal compartments of the skin substitute.

MATERIALS AND METHODS

Cell culture and clonal growth assay Human keratinocytes and fibroblasts were isolated simultaneously from surgical discard tissue using selective growth media and cryopreserved at passage 1 for these experiments (Boyce and Ham, 1983; 1985). For the clonal growth assays, human keratinocytes were inoculated at 25 cells per cm^2 into the test conditions ($n = 4$) and cultured at 37°C and 5% CO_2. The basal medium consisted of modified MCDB 153 (Pittelkow and Scott, 1986) supplemented with 0.5 μg per ml hydrocortisone and penicillin-streptomycin-Fungizone. Human recombinant IGF-I (Peprotech, Rocky Hill, NJ) was tested at 1–30 ng per ml in the presence and absence of 1 ng per ml human recombinant epidermal growth factor (EGF) (Gibco, Rockville, MD). The media were changed four times during the 12 d culture period and the cell colonies were stained at the end of the experiment with 0.1% crystal violet prepared with 2% glutaraldehyde in 0.05 M sodium cacodylate buffer. After 10 min of staining, excess crystal violet stain was removed from the dishes by rinsing with tap water and the dishes were air dried. The crystal violet was released with 1% sodium dodecyl sulfate and the absorbance at 590 nm was measured with a microplate reader (Model 7520 Cambridge Technologies, Watertown, MA). The optical density correlates directly with cell number.

CSS preparation The CSS ($n = 3$ per condition) were prepared by sequential inoculation with human fibroblasts (0.5×10^6 per cm^2) and human keratinocytes (1.0×10^6 per cm^2) onto acellular collagen-GAG biopolymer substrates as previously described (Boyce *et al*, 1991) with the following modifications. The CSS medium consisted of Dulbecco's modified Eagle's medium (1.8 mM Ca^{2+}) supplemented as reported by Chen with modifications (Chen *et al*, 1995). Insulin (0, 0.5, or 5 μg per ml) or IGF-I (0, 10, or 50 ng per ml) was added beginning on day 0 (day of human keratinocyte inoculation onto the collagen substrate). The negative control was represented by CSS cultured in medium with 0 μg per ml insulin + 0 ng per ml IGF-1. On day 3 after human keratinocyte inoculation, the CSS were lifted and maintained at the air–liquid interface in saturated relative humidity at 37°C and 5% CO_2 (Boyce and Williams, 1993). Test media were replaced daily for 4 wk. Samples were taken weekly for histologies, biweekly for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, and at the 4 wk time point for 5-bromo-2′-deoxyuridine (BrdU) incorporation. For light microscopy CSS biopsies were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate buffer, embedded in glycol-methacrylate, sectioned, and stained with Toluidine Blue.

MTT assay On days 14 and 28, 6 mm punch biopsies ($n = 3$ CSS per condition; 12–18 punches per condition) were taken from each CSS condition and incubated for 3 h at 37°C with 0.5 mg per ml MTT (Sigma, St. Louis, MO). The mitochondria of viable cells cleave the tetrazolium salt MTT to formazan (Mosmann, 1983). The MTT formazan reaction product was released by incubating the biopsies in 2-methoxyethanol for 3 h on a rotating platform. The optical density of the MTT formazan product was read at 590 nm on a microplate reader.

BrdU incorporation One square centimeter CSS biopsies were incubated for 22 h with 65 μM BrdU (Roche Molecular Biochemicals, Indianapolis, IN) in a lifted format on day 28. The BrdU-labeled tissue was fixed in 10% buffered neutral formalin and processed for paraffin embedding. The tissue sections were deparaffinized and rehydrated in graded alcohols. Three 0.01 M phosphate-buffered saline washes were performed between the labeling steps outlined below and all solutions were prepared in the same buffer. The CSS sections were partially digested with 0.025% trypsin for 20 min at 37°C followed by 2% bovine serum albumin to inactivate the trypsin. The cellular DNA was denatured with 1.5 N HCl for 15 min at 37°C and then neutralized with 0.1 M sodium tetraborate. Following a blocking step, the sections were incubated with murine anti-BrdU fluorescein isothiocyanate (FITC) (Becton Dickinson, San Jose, CA) at 4°C overnight. Rabbit antipancytokeratin (Zymed Laboratories, South San Francisco, CA) was added to the CSS sections for 90 min and then followed with goat antirabbit IgG-Texas Red antibody (Southern Biotechnology Associates, Birmingham, AL). The slides were coverslipped with Fluoromount G (Southern Biotechnology Associates). The BrdU-positive human keratinocytes were determined by counting the number of BrdU-FITC positive/pancytokeratin-Texas Red positive cells per field (field length 550 μm). Four to six unique sections were counted per CSS ($n = 3$ CSS per condition) at the day 28 time point.

Immunohistochemistry CSS biopsies 0.25 cm^2 (n = 6 CSS) were processed for cryostat sectioning by freezing in M-1 embedding matrix (Shandon-Lipshaw, Pittsburgh, PA). The 15 μm CSS sections were fixed in acetone and rehydrated in 0.05 M Tris-buffered saline, pH 7.5. Multiple washes with 0.05 M Tris-buffered saline/0.05% Tween-20 took place between each step in humidified chambers at room temperature. Following a blocking step, the sections were incubated in 10 μg per ml murine antihuman IGF-I (Serotec, Raleigh, NC) or 10 μg per ml mouse IgG negative control (Serotec) for 30 min. The biotinylated secondary antibody (Vectastain Universal Elite ABC kit; Vector Laboratories; Burlingame, CA) was pipetted onto the CSS sections. Endogenous peroxidase activity was inhibited with a 0.3% hydrogen peroxide incubation step. The slides were incubated with the Vectastain Elite ABC reagent, which is a mixture of avidin and biotinylated horseradish peroxidase, for 30 min. Biotinyl tyramide (Renaissance Tyramide Signal Amplification-Indirect kit; NEN Life Science Products, Boston, MA) was utilized to enhance the IGF-I signal. The biotinyl tyramide amplification system employs horseradish peroxidase to catalyze biotin-labeled tyramide deposition at the site of the horseradish peroxidase enzyme. Streptavidin-horseradish peroxidase was incubated with the tyramide-labeled sections followed by diaminobenzidine tetrahydrochloride (Vector Laboratories), the peroxidase substrate. The tissue sections were dehydrated, cleared, and mounted with permount.

***In situ* Hybridization** CSS biopsies 0.25 cm^2 (n = 3 CSS) were fixed in 4% paraformaldehyde for 20 h at room temperature and processed for paraffin embedding. To reduce the ribonuclease contamination, all solutions were prepared with diethyl-pyrocarbonate-treated, autoclaved water and multiple washes were performed between each step. Tissue sections were deparaffinized in xylene and rehydrated in graded alcohols and 0.01 M phosphate-buffered saline. Postfixation was carried out with 4% paraformaldehyde and the sections were treated sequentially with 10 mg per ml glycine, 0.2% tritonX-100, and 3 μg per ml proteinase K for 60 min at 37°C and then postfixed with 4% paraformaldehyde. The slides were washed with 0.1 M triethanolamine and 0.1 M triethanolamine/0.25% acetic anhydride to acetylate the tissue. CSS sections were dehydrated in graded alcohols and air dried for 1 h.

Prehybridization and hybridization occurred in the following solution: 4 × saline–sodium citrate buffer (SSC), 0.3% sodium pyrophosphate, 0.6% polyvinyl pyrrolidone (mw 40,000), 0.6% ficoll (mw 400,000), 5 mg per ml acetylated bovine serum albumin, and 0.1 mg per ml salmon sperm DNA. The CSS tissue sections were prehybridized at 55°C in humidified chambers for 90 min. The hybridization solution consisted of 1 ng per μl digoxigenin (DIG)-labeled IGF-I antisense or sense riboprobes that had been denatured for 5 min at 80°C and incubated overnight with the CSS sections at 55°C. The riboprobes were produced by linearization of a cloned IGF-I plasmid (ATCC, Rockville, MD) and DIG transcription labeling using a DIG RNA Labeling kit (Roche Molecular Biochemicals) (Brown *et al*, 1997).

Posthybridization included multiple wash steps with 0.5 × SSC/1 mM ethylenediamine tetraacetic acid (EDTA) at 55°C, 50% formamide/0.15% sodium chloride/5 mM Tris-HCl/0.5 mM EDTA, 0.5 × SSC, and 0.2 × SSC at 55°C. The tissue sections were incubated with 20 μg per ml RNAse A and 10 units per ml RNAse T_1 for 30 min at 37°C. Blocking with 1% serum (Roche Molecular Biochemicals) took place at room temperature for 90 min. The CSS sections were incubated overnight at 4°C with an alkaline phosphatase conjugated anti-DIG antibody (Roche Molecular Biochemicals) diluted at 1:750 in blocking buffer. On the following day, the sections were exposed to the alkaline phosphatase substrate, BM purple (Roche Molecular Biochemicals), in the dark for 1–2 h at room temperature and preserved with Crystal Mount (Biomeda, Foster City, CA). Specificity of the *in situ* hybridization reaction was confirmed by the absence of hybridization of the antisense probe following pretreatment of CSS sections with RNAse A (200 μg per ml, 2.5 h, 37°C).

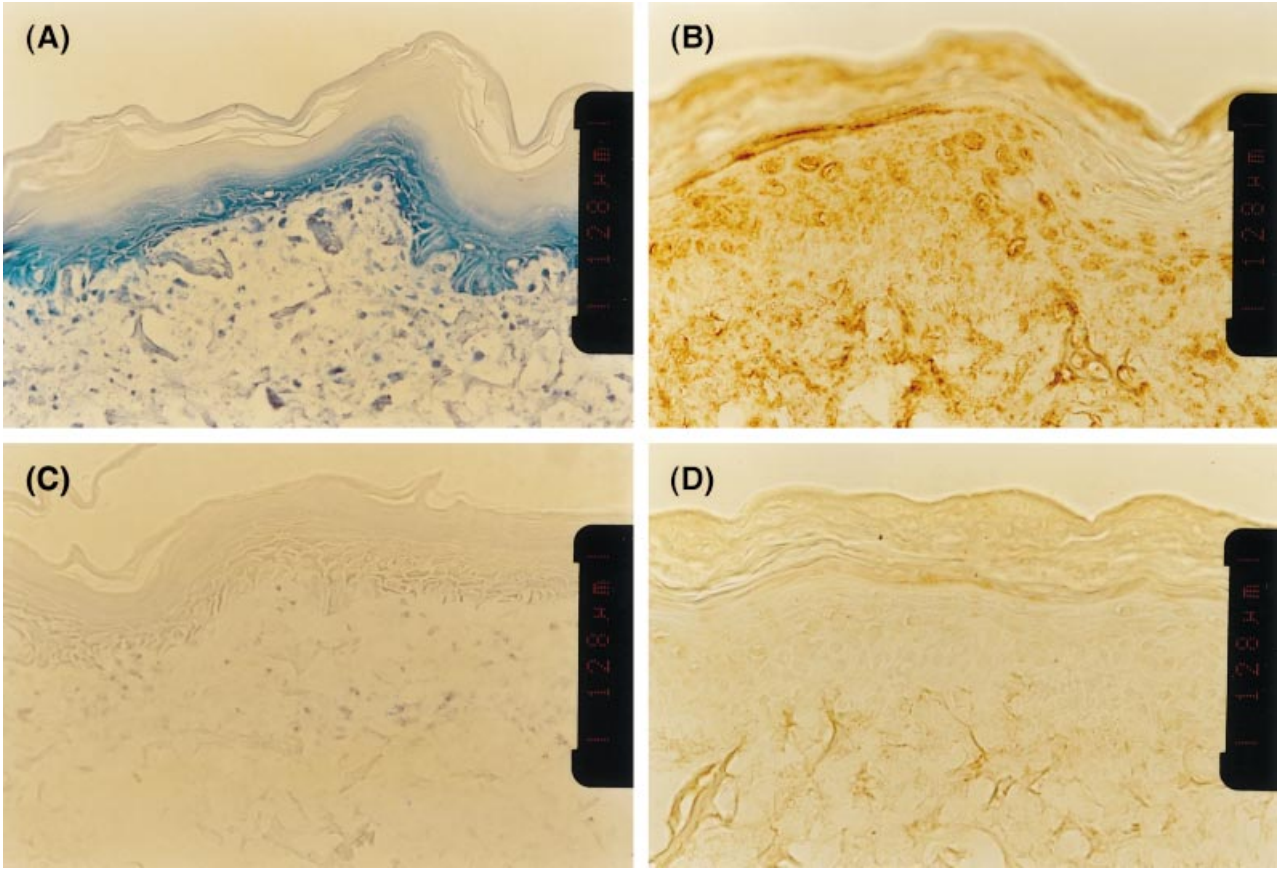

Figure 1. Cultured skin substitues (CSS) express mRNA and protein for insulin-like growth factor-I (IGF-I) after 21 d incubation *in vitro*. (*A*) *In situ* hybridization of IGF-I anti-sense riboprobe to CSS showing strong labeling in nucleated keratinocytes of the epidermal component, and uniform staining in fibroblasts in the dermal component. (*B*)Immunohistochemical staining with anti-IGF-I monoclonal antibodies shows positive staining for human IGF-I protein in both the epidermal and the dermal components of CSS. (*C*) *In situ* hybridization of IGF-I sense riboprobe to CSS showing no labeling in CSS. (*D*) No staining is observed with murine IgG as the primary antibodies. Scale = 0.128 mm.

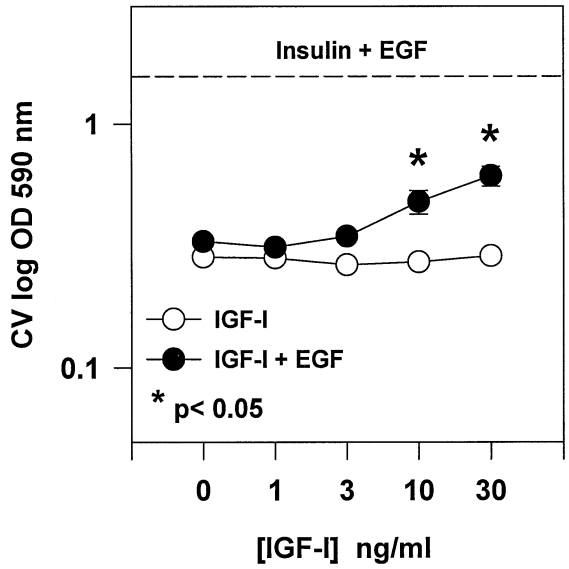

Figure 2. Growth of cultured human keratinocytes responds to IGF-I as a function of concentration in the presence of epidermal growth factor (EGF). The positive control condition of insulin (5 μg per ml) and EGF (1 ng per ml) (- - - -) stimulates maximum growth (1.1 OD_{590} units) of keratinocytes in the clonal growth assay. Substitution of IGF-I at 10 or 30 ng per ml (●) with EGF stimulates statistically significant increase of growth compared with IGF-I without EGF (○), but remains significantly less than insulin and EGF. (*p<0.05 *versus* all other conditions).

Surface electrical capacitance (SEC) SEC represents a measure of skin surface hydration, which is inversely proportional to the electrical impedance (Boyce *et al*, 1996; Supp *et al*, 1999). The NOVA Dermal Phase Meter (DPM 9003; NOVA Technology, Gloucester, MA), which is based on electrical impedance, was used to measure the SEC on the CSS *in vitro*. The application of a current between two electrodes at the skin surface enables the NOVA meter to detect an electrical phase shift over time. The magnitude of the phase shift increases as water accumulates at the site of the capacitance probe, with corresponding increase in the SEC values. Therefore, CSS with intact barrier yield low SEC values and CSS with poor barriers result in high SEC readings.

Ten serial readings at 1 s intervals were recorded from each CSS on culture days 7, 10, 14, 17, 21, 24, and 28. Six SEC measurements were taken on each of three CSS per condition at each time point (18 SEC values per treatment group). The SEC data are expressed in picofarads as mean ± SEM.

Statistical analysis The data represent the mean ± SEM for each condition. The human keratinocyte clonal growth experiments, MTT assay, and BrdU incorporation data were analyzed by a one-way ANOVA. The method used for multiple comparisons was Student–Newman–Keuls and significance was established at the 95% confidence level ($p < 0.05$).

RESULTS

IGF-I expression by CSS CSS were incubated *in vitro* for 28 d including 25 d at the air–liquid interface. Paraffin sections were prepared for *in situ* hybridization at weekly intervals. The collagen-GAG substrate contains numerous fibroblasts uniformly spaced throughout the collagen sponge, and a well organized epithelium and stratum corneum (**Fig 1**). The IGF-I antisense riboprobe

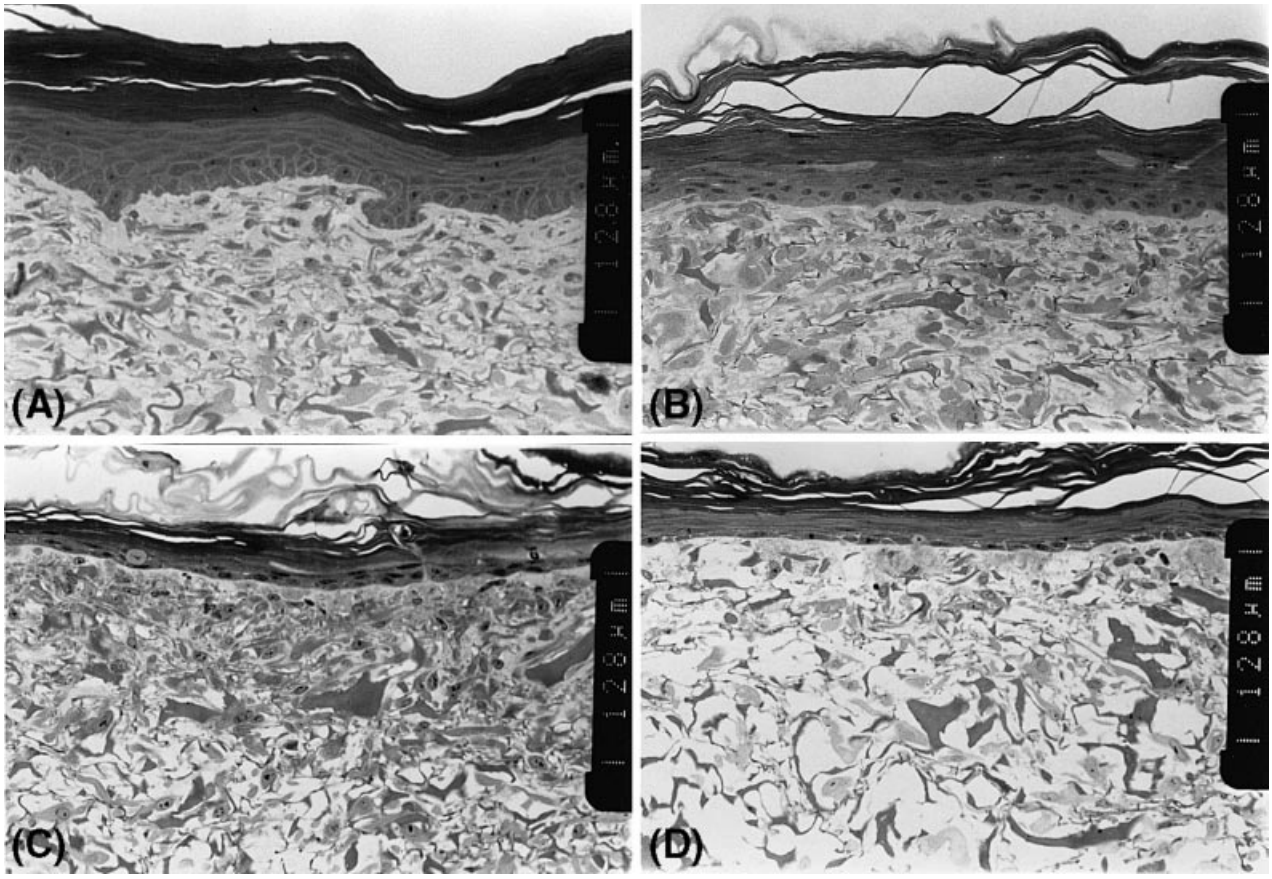

Figure 3. Epidermal morphology of CSS depends on insulin after 28 d incubation *in vitro*. Insulin, but not IGF-I, supports full stratification and differentiation of the epidermal component of CSS, including an analog of the stratum corneum. (*A*) Insulin at 5 μg per ml, (*B*) insulin at 0.5 μg per ml, (*C*) IGF-I at 50 ng per ml generated a thin epithelium with fewer layers of nucleated or cornified keratinocytes. (*D*) Incubation with 0 μg per ml insulin + 0 ng per ml IGF-I also generated a very thin epithelium with few nucleated layers, similar to (*C*). Scale = 0.128 mm.

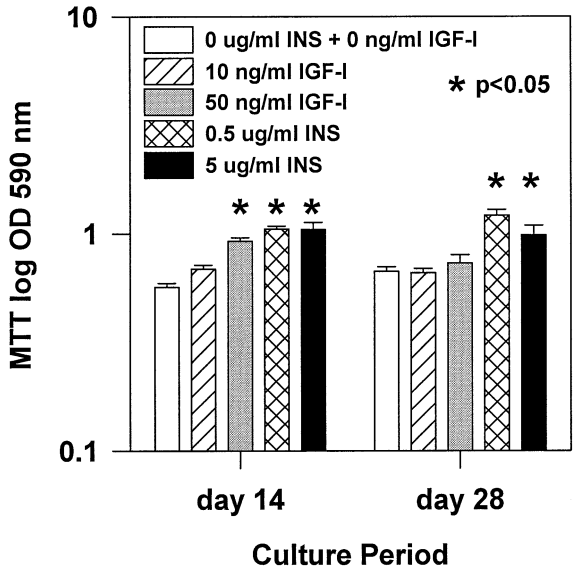

Figure 4. Insulin stimulates greater mitochondrial metabolism than IGF-I after 14 or 28 d of incubation of CSS. At 14 d of incubation, insulin at 5 μg per ml or 0.5 μg per ml, or IGF-I at 50 ng per ml stimulated significantly greater MTT conversion than 0 μg per ml insulin plus 0 ng per ml IGF-I, or 10 ng per ml IGF-I (*p<0.05). At 28 d of incubation, 5 μg per ml or 0.5 μg per ml insulin stimulated significantly greater MTT conversion than IGF-I at 10 ng per ml or 50 ng per ml, or 0 μg per ml insulin and 0 ng per ml IGF-I (*p<0.05).

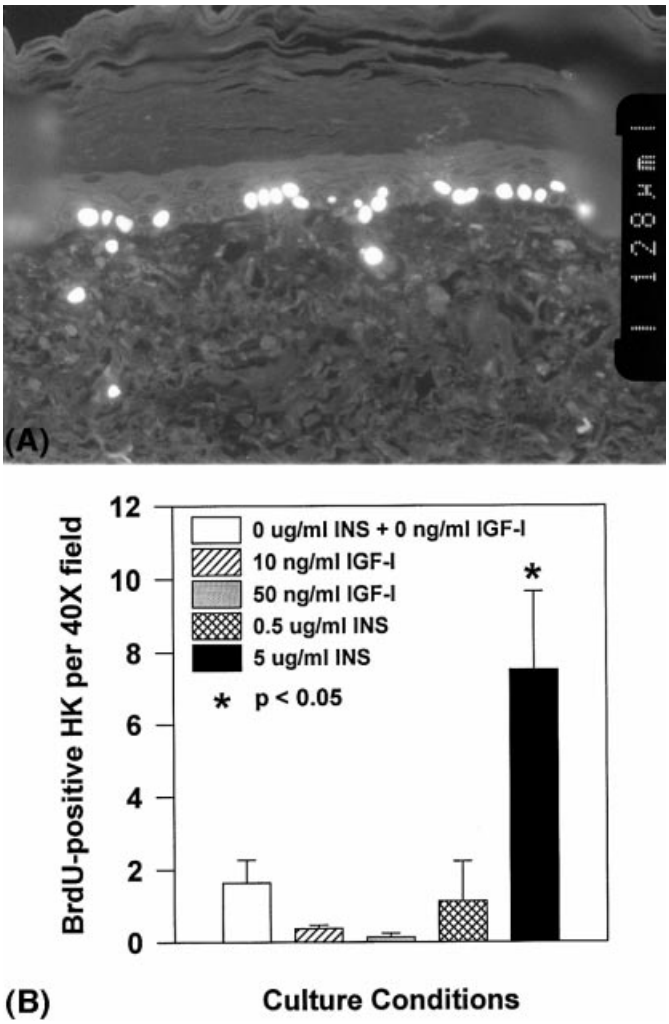

Figure 5. Insulin stimulates greater DNA synthesis than IGF-I at 28 d of incubation of CSS. (*A*) BrdU labeled keratinocytes and fibroblasts occur frequently in CSS incubation in insulin. Scale = 0.128 mm (*B*) Insulin at 5 μg per ml stimulates significantly greater incorporation of BrdU than other conditions tested (*p<0.05).

hybridized strongly with the epidermal cells and fibroblasts of the CSS (**Fig 1*A***). The DIG-labeled sense probe served as the negative control as shown in **Fig 1*C*** with minimal background evident. The IGF-I message was expressed at weeks 1 through 4 of *in vitro* culture.

The ability of the CSS to synthesize IGF-I protein *in vitro* was confirmed using immunohistochemical techniques. The epidermis and dermis of the CSS stained positively for the presence of IGF-I at all time points (**Fig 1*B***). No staining was observed after reaction of CSS with nonspecific murine IgG as the primary antibody (**Fig 1*D***). The intensity of the diaminobenzidine staining appeared greatest at the day 21 time point, indicating a peak in IGF-I synthesis in CSS maturation.

IGF-I stimulation of keratinocyte proliferation Human keratinocyte proliferation was stimulated significantly by IGF-I at 10 or 30 ng per ml plus 1 ng per ml EGF (0.48 ± 0.05 or 0.61 ± 0.06 OD_{590}, respectively) compared to IGF-I at 10 or 30 ng per ml without EGF (0.27 ± 0.01 or 0.29 ± 0.09 OD_{590}, respectively) (**Fig 2**). At the concentrations tested, IGF-I did not substitute fully for insulin at 5 μg per ml plus 1 ng per ml EGF. Keratinocyte proliferation was not stimulated by either insulin (5 μg per ml) in the absence of EGF (data not shown), or IGF-1 (1–30 ng per ml).

IGF-I substitution for insulin in growth medium of CSS In **Fig 3*A***, light microscopy of CSS at day 28 shows that the CSS grown in 5 μg per ml insulin had abundant epithelial cell layers and stratum corneum. Incubation in 0.5 μg per ml insulin generates CSS with variable morphology with fewer layers of nucleated and cornified keratinocytes (**Fig 3*B***). Culture medium with 50 ng per ml IGF-I resulted in poor CSS morphology (**Fig 3*C***) with few layers of nucleated human keratinocytes and minimal stratum corneum. At day 28, there was no morphologic difference between the 50 ng per ml IGF-I CSS and the 0 μg per ml insulin + 0 ng per ml IGF-I negative control (**Fig 3*D***). At the day 14 time point, the 10 ng per ml IGF-I and negative control conditions had a poorer morphology than the other conditions. The 50 ng per ml IGF-I condition at day 14 more closely resembled the insulin-containing groups histologically (data not shown), but deteriorated by day 28.

Culture conditions with either 0.5 or 5 μg per ml insulin (1.05 ± 0.03 or 1.05 ± 0.08 OD_{590}, respectively), or 50 ng per ml IGF-I (0.92 ± 0.04 OD_{590}), generated significantly greater MTT formazan *versus* the other culture conditions at day 14 (**Fig 4**). By day 28 of *in vitro* culture, the MTT formazan values from CSS grown in the negative control medium (0.67 ± 0.03 OD_{590}) and IGF-I at 10 or 50 ng per ml (0.66 ± 0.03 or 0.74 ± 0.07 OD_{590}, respectively) were significantly lower than those from CSS cultured with 0.5 or 5.0 μg per ml insulin (1.21 ± 0.08 or 0.98 ± 0.10 OD_{590}, respectively). The MTT data from the 50 ng per ml IGF-I condition were not statistically different at day 14 from the insulin-treated CSS, but had significantly lower MTT values by day 28, suggesting cellular degeneration in the CSS.

BrdU labeling of epidermal cells Double labeling for DNA synthesis and intracellular keratin allowed for the identification of BrdU-positive keratinocytes in the CSS at day 28 (**Fig 5*A***). The BrdU incorporation was almost exclusively confined to basal human keratinocytes in the epidermal component and a small number of fibroblasts in the dermal compartment of the CSS. At day 28 of *in vitro* culture, BrdU labeled a significantly greater

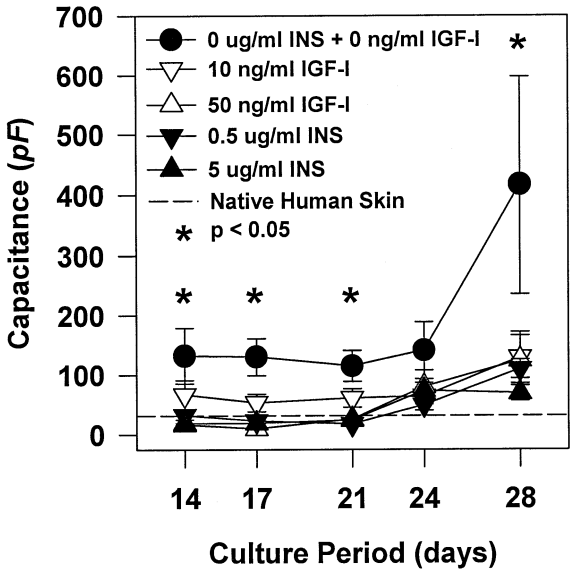

Figure 6. Epidermal barrier forms in CSS incubated in insulin or IGF-I. Surface electrical capacitance (pF) in CSS decreases to values not different from native human skin (NHS) (- - - -) from 14 to 28 d of incubation in 5 μg per ml (▲) or 0.5 μg per ml (▼) insulin, or 10 ng per ml (▽) or 50 ng per ml (△) IGF-I. Incubation of CSS in 0 μg per ml insulin and 0 ng per ml IGF-I generated CSS with statistically wetter surfaces than other groups, or than NHS (*$p<0.05$).

number of basal keratinocytes incubated with 5 μg per ml insulin (7.51 ± 2.15 human keratinocytes per field) than all other groups (**Fig 5*B***), including the 0.5 μg per ml insulin condition (1.14 ± 1.10 human keratinocytes per field).

Epidermal barrier in CSS incubated with IGF-I In **Fig 6**, SEC was the same for all conditions at the day 7 and day 10 time points (data not shown). The SEC values were statistically higher for the negative control lacking insulin and IGF-I at day 14, 17, 21, and 28, indicating poor barrier development. SEC values for all other treatment groups were not statistically different from each other, nor from values for native human skin of normal volunteers.

DISCUSSION

IGF-I is an important autocrine/paracrine factor for the growth and function of both keratinocytes and fibroblasts in the skin (Nickoloff *et al*, 1988; Barreca *et al*, 1992; Kratz *et al*, 1992). These studies were designed to elucidate the physiologic role of IGF-I in the growth and maturation of CSS. Using immunohistochemistry and *in situ* hybridization, IGF-I protein and mRNA were detected in the CSS during the 4 wk culture period (**Fig 1**). Several studies have noted the presence of IGF-I in the epidermis of normal human skin (Rudman *et al*, 1997; Tavakkol *et al*, 1999; Blakytny *et al*, 2000), and injured murine epidermis exposed to ultraviolet B irradiation was positive for IGF-I (Hansson *et al*, 1988). Also, IGF-I was present in the regenerating epithelial wound edge of mice (Brown *et al*, 1997). The CSS represents rapidly proliferating human keratinocytes and human fibroblasts similar to a healing wound and IGF-I was identified throughout the epidermal and dermal components of the CSS.

IGF-I alone did not support human keratinocyte clonal growth in the absence of EGF (**Fig 2**). These results were consistent with the poor stimulation of human keratinocyte growth provided by insulin-containing medium deficient in EGF (data not shown). If EGF was added to the human keratinocyte culture medium, insulin (5 μg per ml) stimulated the keratinocyte growth to a significantly greater degree than IGF-I (1–30 ng per ml). The increased growth of human keratinocytes in the presence of insulin together with EGF in the culture medium suggests that optimal human keratinocyte responses were mediated through receptors for both mitogens. Because only insulin promotes glucose transport, however, it is possible that the increased mitogenic response results from increased availability of energy to the cells as well as initiation of DNA synthesis. Conversely, in the absence of insulin, glucose supply may be rate limiting to the stimulation of mitosis by IGF-I. Other studies have reported that the *in vitro* mitogenic effects of IGF-I on human keratinocytes are similar to or greater than those of insulin (Nickoloff *et al*, 1988; Neely *et al*, 1991; Barreca *et al*, 1992; Eming *et al*, 1996). In those experiments, other growth factors were included in the culture media and higher human keratinocyte densities were used to perform the growth assays. The results reported here agree well with a study by Krane *et al* (1991) where EGF was necessary to measure a positive mitogen response to IGF-I by human keratinocytes. Both of these investigations were performed in basal culture conditions containing only hydrocortisone. Under the more stringent conditions of the clonal growth assay, IGF-I substituted only partially for insulin in the stimulation of human keratinocyte growth.

Neither the endogenous IGF-I produced by CSS nor the addition of IGF-I to the CSS culture medium was adequate to replace the insulin requirements for optimal CSS anatomy and sustained cellular proliferation (**Figs 3**, **4**). Light microscopy and MTT measurements revealed that the CSS incubated in the higher IGF-I concentration were similar to the insulin treated CSS at day 14. By day 28 significant anatomic deterioration was observed in CSS incubated only in IGF-I, and this was indistinguishable from the negative control CSS incubated in the absence of both insulin and IGF-I. These results support the hypothesis that insulin cannot be replaced by IGF-I in the physiologic development of CSS. BrdU incorporation of the CSS showed that by day 28 the 5 μg per ml insulin treated CSS had significantly higher BrdU labeling than all other treatment groups (**Fig 5**). The CSS condition with 0.5 μg per ml insulin had significantly fewer BrdU-positive human keratinocytes than the CSS with 5 μg per ml insulin, which demonstrated that epidermal proliferation slowed significantly by day 28. This result was consistent with the light microscopy, but not with data from the MTT assay with CSS in low dose insulin. These data support an absolute requirement for insulin to generate CSS with sustained mitochondrial metabolism and DNA synthesis (**Figs 4**, **5**). The data also suggest, however, that a lower concentration of insulin (0.5 μg per ml) is sufficient to maintain mitochondrial metabolism (**Fig 4**), but that a higher concentration of insulin (5 μg per ml) is required to stimulate mitosis for extended periods of time (**Fig 5**). Higher insulin requirements for mitosis may correspond to higher energy requirements for cell division compared to G0/G1 interphase metabolism.

Epidermal barrier by measuring CSS SEC was less well developed in the negative control lacking both insulin and IGF-I (**Fig 6**). Insufficient supplies of glucose for lipid synthesis also account for poor formation of epidermal barrier by CSS incubated in medium without insulin (**Fig 6**). No statistical difference was noted between the other treatment groups. The SEC data were not supported by the CSS light microscopy and MTT data (**Figs 3**, **4**) inasmuch as IGF-I supplemented media resulted in poor CSS morphology but had low surface hydration as measured by SEC. It is probable, however, that the keratinocytes incubated in IGF-I produced stratum corneum early in CSS development from residual insulin carried from the proliferating human keratinocytes used to prepare the skin substitutes. As culture time progressed, the epidermal cells deteriorated in the media lacking insulin although the stratum corneum remained intact in the absence of proliferating human keratinocytes beneath. Therefore, SEC data must be interpreted as only a measure of surface hydration that indicates the formation of epidermal barrier but does not indicate the proliferative capacity of keratinocytes at the time that the surface hydration is measured. Because keratinocyte proliferation is required for barrier formation, proliferation is the critical factor

for stable wound healing. With this consideration, SEC data should be combined with an assessment of cellular proliferation to estimate the efficacy of wound healing. It may be expected that CSS with both proliferating keratinocytes and epidermal barrier would provide greater efficacy of wound healing than proliferating keratinocytes without barrier, and that functional stratum corneum without proliferating keratinocytes would not heal a wound.

Insulin and IGF-I can bind to each other's receptors implying that these factors may have overlapping functions. Experiments by Eming *et al* (1996) showed that human keratinocytes genetically modified to overexpress IGF-I did not require insulin for proliferation. This result may be attributed, in part, to the extremely high levels of IGF-I secreted by the modified cells and a shorter assay period than reported here. Those investigators also grafted the epithelial sheets from IGF-I modified and unmodified human keratinocytes onto athymic mice with comparable *in vivo* morphology. *In vitro*, IGF-I has been reported to be more potent than insulin in stimulating human keratinocyte proliferation (Neely *et al*, 1991). IGF-I is the only other factor with hypoglycemic effects similar to insulin, yet IGF-I and insulin have very distinct physiologic functions (LeRoith *et al*, 1994; Sherwin *et al*, 1994). The studies presented here demonstrate that CSS synthesize IGF-I *in vitro* although the endogenous production of IGF-I was not adequate to support CSS development in the absence of insulin. Also, the addition of IGF-I to the culture medium could not reproduce the insulin-dependent CSS morphology. These experiments support very strongly the hypothesis that the growth and maturation of CSS requires insulin. It is expected that greater homology of CSS with the anatomy and physiology of healthy human skin will lead to reductions in mortality and morbidity from full-thickness cutaneous wounds.

These studies were supported by grants from the National Institutes for Health (GM50509), and the Shriners Hospitals for Children (#8670 and #8450) to Dr. Boyce. The authors thank Jodi Miller, Todd Schuermann, Christine Kane, and Gail Macke for expert technical assistance with these experiments.

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